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TECHNIQUES FOR THE STUDY OF INSECT CHROMOSOMES*

BY STANLEY G. SMITH,
Montreal, Quebec

I. PREPARATION METHODS.

INTRODUCTION.

The present paper is concerned solely with methods of studying the chromosomes, their number, morphology, and behaviour, in insects. Much has already been written on general cytological techniques used in the study of animals and plants (1, 2, 3 and 4) but no special treatment regarding insects has as yet appeared. The techniques outlined in many standard text books are largely obsolete, and reprints of current improvements are frequently not available in the specialised entomological library. Therefore a review of the procedures commonly followed may not be out of place as a guide to the non-specialist.

It should be pointed out that partly because of the inaccessibility of many of the journals from which many of the data to follow have been collected, and partly because this paper is intended to be self-sufficient, but mainly for economy in space, credit to the various authors has in most cases been omitted.

CHOICE OF TISSUES

In general, characteristics of taxonomic value such as relative size and the position of primary and secondary constrictions, are determinable only in somatic chromosomes. The number, however, can be determined during both mitosis and meiosis. Clearly, in either case, it is necessary to examine cells during their time of active division, and such cells are usually to be found only during periods of active growth.

Insects taken before the last moult can usually be relied upon to supply greater or lesser numbers of dividing cells in such tissues and organs as the mesenteron, proctodaeum, hypodermis, fat body, ganglia, tracheal tubes, follicles of the gonads, connective tissues and intestinal muscles. In holometabalous forms the wing-buds, limb-buds and brain are usually rich in divisions during the prepupal-pupal metamorphosis. The early embryonic stages, of course, will show numerous dividing cells, but the chromosomes are frequently small and rarely amenable to study.

In the case of meiosis the times in the life history at which the various stages are to be found are less uniform. In the male, of course, the maturation divisions are completed before copulation, but they may occur either before or during adult life. In the female, on the other hand, the actual maturation divisions are more frequently undergone after copulation and even after oviposition, although in some adult coccids the egg may develop as far as the early gastrula stage before being laid. It is therefore advisable to ascertain whether divisions are occurring by making a preliminary examination using a combined fixing and staining solution such as aceto-carmine (see below).

DISSECTION.

The method used in dissection is dependent on the subsequent treatment. There are four main treatments in general use which all attain the same end, that is, the separation of a mass of cells, more or less impervious to light, into a

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transparent layer one or a few cells in thickness. The four involve a) combined fixing and staining, b) embedding and sectioning, c) smearing, and d) squashing.

With embedding in mind and using a subject of relatively large size it is usually best to expose the body cavity and transfer the tissue or organ directly into a vial containing about 3 cc. of fixative. This results in rapid fixation since the fixative is not appreciably diluted. On the other hand, if small organs such as gonads are sought, it is often advisable to inject the fixative forcibly among the viscera. By this means the enveloping materials are floated away, leaving the organs freely exposed and easily dissected. A further advantage in the case of gonads is that the fixative changes them from transparent to opaque white (or the colour of the fixative) and thereby makes them simpler to locate.

If, as is usual, testes are to be smeared, they should be dissected by flooding with frog Ringer or an isotonic saline solution (.75-.90 per cent NaCl), as prefixation will result in coagulation of the contents, which prevents successful smearing.

Both the squash method and the aceto-carmine method are possible following fixation *in situ*, but to prevent hardening of the tissue both pre- and post-fixation should be shortened to about 10-15 minutes. Neural ganglia, whole brains, wing-buds, limb-buds and various tissues are best treated in this way.

The small gonads of early instars, which are difficult to find in the enveloping sheets of fat body, are best removed by making a dorsal incision in the body wall and squeezing from the ventro-lateral sides; the exuded fat body with contained gonads is then transferred to the saline or fixative and shaken vigorously. The fat-body cells fall apart and liberate the gonads.

Fully mature or almost mature eggs and embryos can usually be transferred directly to the fixative; they may be embedded and sectioned, squashed, or preferably stained and mounted *in toto* (see later).

THE ACETO-CARMINE METHOD.

A saturated solution of carmine in 45 per cent acetic acid fixes and stains chromosomes simultaneously. It was originally designed by Schneider in 1880 for the study of animal material but has since been adapted to the study of plant chromosomes. The modifications introduced by plant workers when applied to animals have led to considerable improvement and have proved especially valuable for the study of salivary gland chromosomes (see later).

Aceto-carmine may most simply be prepared in the following manner: an excess of carmine (about 1/4 gm. or more) is added to 50 cc. of boiling 45 per cent acetic acid, simmered for 2-3 minutes, cooled on ice and filtered.

The simplest use of aceto-carmine consists merely in teasing or squashing the material in a drop of the solution, and covering with a glass slip. If the preparation is satisfactory, it can be sealed by means of a mixture of gum mastic and paraffin applied to the edges of the coverslip with a hot needle. The differentiation between the chromosomes and the cytoplasm is usually improved if the slide is then stored in a refrigerator and allowed to "ripen". Once the desired result has been attained, the aceto-carmine should be replaced with 45 per cent acetic acid drawn under the coverslip by filter paper; this prevents overstaining.

There are various modifications of the aceto-carmine method which appropriately combined produce results far superior to the simple method:

a) Prefixation in alcohol-acetic (absolute alcohol 3 and glacial acetic acid 1, freshly mixed) allows less swelling of the chromosomes and gives sharper definition, besides serving as a dissecting fluid. After 10-15 minutes fixation mount directly in aceto-carmine or store in 70 per cent alcohol.

b) Heating the material either in bulk (at 60° C) or after spreading on the slide swells the chromosomes, gives sharper differentiation and intensifies the

stain. Heat the slide over an alcohol flame four or five times, taking care to prevent the fluid from boiling. If between successive heatings pressure is applied to the coverslip through a layer of filter paper, the cells will be separated and flattened and the chromosomes will be spread out.

c) Overstaining may be reduced by replacing the aceto-carmine by 45 per cent acetic acid and heating.

d) The addition of iron to the stain gives a more intense stain and better differentiation (Belling's iron-aceto-carmine). The iron may be added in the form of a ferric salt to the bulk stain or to the alcohol-acetic used in prefixation. Too much iron will cause the cytoplasm to stain and hinders the maximum spreading of the chromosomes. It is usually better to macerate the material in a drop of aceto-carmine on the slide, stir a second drop on the coverslip with a steel needle until it turns faintly bluish, unite the slide and coverslip, heat immediately and apply considerable pressure. For even sharper differentiation and extreme spreading, as is needed with prophase stages, the material is best macerated in straight 45 per cent acetic acid, adding the ironized stain to the coverslip and applying with pressure; this, however, requires that the stage of the material be predetermined.

e) Maceration is sometimes necessary to attain complete spreading with certain materials, and is carried out by using hot acetic acid or hydrochloric acid. In the former case bulk material in 45 per cent acetic acid or aceto-carmine is placed in an oven at 60° C; in the latter prefixed material is transferred to a mixture of equal parts of 95 per cent alcohol and concentrated HCl for 5-10 minutes, returned for a short time to alcohol-acetic and squashed in aceto-carmine.

f) Satisfactory preparations can be made permanent by the McClintock method as follows:—place the slide flat in a dish of 10 per cent acetic acid; when the coverslip floats free insert it in the split end of a match and run both the cover and the slide through the following solutions (leaving them in each for a few minutes):— absolute alcohol 3, glacial acetic 1; absolute alcohol 9, acetic 1; two changes of absolute alcohol; absolute alcohol 1, xylol 1. Add a drop of Canada balsam to the coverslip, reunite with the slide as nearly as possible in its original position, press down and remove excess balsam. The union of the coverslip and slide should be as rapid as possible to prevent the absorption of moisture by the alcohol, since this leads to fogging. For floating off the coverslip a mixture is sometimes used consisting of equal parts of glacial acetic acid, absolute alcohol, and xylol. It is followed by two changes in absolute alcohol-xylol before mounting in balsam.

g) If difficulty is experienced in getting the cells to adhere to the slide, it can be overcome in the following manner:—smear a fine film of Mayer's albumen (filtered mixture of white of egg 50 cc., glycerine 50 cc. and camphor 1 gm.) on the slide, heat until a white vapour is given off and cool before using. It is perhaps advisable to use this method always as all the material adheres to the slide, thus saving the trouble of running up the coverslip and because a fresh coverslip with balsam can be applied the moment the slide is removed from the absolute-xylol mixture, thus reducing the possibility of fogging by moisture.

Acetic-orcein (2 gm. orcein, 2 gm. glacial acetic acid and 100 cc. distilled water) was originally (1886) said to stain nuclei blue and cytoplasm pink. It has recently been shown that this stain can be used as a substitute for aceto-carmine. To 45 cc. of boiling glacial acetic acid add 1 gm. orcein, and when cold add 55 cc. distilled water, shake and filter. It is used in the same way as aceto-carmine. Should maceration be necessary, add 1 cc. N HCl to 10 cc. acetic-orcein; for hardening, add 1 cc. chloroform to 10 cc.

THE PARAFFIN METHOD.

i) *Fixation*:

There are four main types of fixatives used in the paraffin technique, namely, chrom-osmic-acetic, chrom-formal-acetic, picric-formal-acetic, and alcohol-acetic (with or without formaldehyde). Of the many modifications of these, the best appear to be La Cour's 2BD, Randolph's CRAF, Allen's B-15, and Kahle's fluid.

Formulae of Fixatives:

2BD:

Chromic acid, 1 per cent	100 cc.
Potassium bichromate, 1 per cent	100 cc.
Saponin	0.1 gm.
Osmic acid, 2 per cent	30 cc.
Acetic acid, 5 per cent	30 cc.

B-15:

Picric acid, sat. aqu. sol.	1 gm.
Formaldehyde	25 cc.
Glacial acetic acid	5 cc.
Chromic acid	1.5 gm.
Urea	2 gm.

N.B. The first three alone constitute the original Bouin's fluid.

CRAF:

A) Chromic acid	1 gm.
Glacial acetic acid	7 cc.
Dist. water	92 cc.
B) Formaldehyde	30 cc.
Dist. water	70 cc.

Mix in equal parts before using.

KAHLE:

Alcohol, 95 per cent	15 cc.
Formaldehyde	6 cc.
Glacial acetic acid	1 cc.
Dist. water	30 cc.

Omit the water for Modified Kahle; replace with sat. aqu. sol. picric acid for Picro-Kahle.

The choice of fixative is determined primarily by the nature of the material and then by the stain contemplated. Though no specific rules can be laid down experience suggests the following: 1) for ovaries use B-15 or Picro-Kahle before haematoxylin; Modified Kahle before the Feulgen stain; and CRAF or 2BD before crystal violet, 2) for testes, when seriation of stages is desired, use CRAF or 2BD before crystal violet and Kahle's before Feulgen; 3) for eggs and young embryos use Modified Kahle before Feulgen, haematoxylin or chromic-crystal violet.

It appears that the lengthy fixations recommended in many early publications are, if not excessive, certainly unnecessary. With small pieces of material fixation is complete in four hours using 2BD, B-15 or CRAF, and in only two or less using Kahle's fluid or modifications of it. With all materials except those fixed in alcoholic fluids, penetration is aided by placing the vial in a vacuum pump for about 3 minutes. In the case of relatively large ovaries penetration of aqueous fixatives can be hastened by placing them first in Carnoy's fluid (absolute alcohol 6, chloroform 3 and glacial acetic acid 1) for 1-2 minutes.

ii) *Washing*:

Certain fixatives require more or less complete removal before dehydration and infiltration. Material in fixatives containing osmic are given several changes in cold water and then soaked in tepid water for about 15 minutes. Kahle-material may go directly into 35 per cent alcohol, and CRAF, B-15 or Modified Kahle-fixed material should be transferred directly into 70 per cent alcohol. This not only saves considerable time but gives better staining, for picric and chromic acids are mordants for haematoxylin and crystal violet respectively, and formaldehyde appears to improve the reaction with the Feulgen stain.

iii) *Dehydration and Infiltration*:

The commonest dehydrant is ethyl alcohol it is used for all forms of dehydration, both before and after embedding, and before and after staining. Other fluids which have recently come into prominence are normal butyl alcohol (and iso-butyl alcohol) and dioxane (diethylene oxide).

Because of its greater bulk, material fixed for embedding requires longer than smears or squash preparations in the successive steps to replace the water by the increasing concentrations of alcohol. The following schedule has been

found satisfactory: 10, 20, 40 per cent, 30 minutes each; 60 per cent, one hour; 70 per cent, three hours (or overnight); 80, 95, and two changes in 100 per cent, 1 hour each.

Since ethyl alcohol is not a solvent of paraffin wax it is necessary to replace it with a fluid capable of dissolving paraffin, such as benzol, xylol or chloroform. The chloroform is more expensive but is to be preferred as it has a lesser hardening effect and is more volatile. The alcohol is gradually replaced by chloroform by running the material through chloroform-alcohol 1:3, 2:2, 3:1, 2 hours in each, and then into pure chloroform. Add a piece of wax about the size of a pea and transfer to the top of an embedding oven (about 30° C); add two or three more pieces over a period of 48 hours; pour into a watch glass and place in a paraffin oven (56° C) for about four hours for final evaporation of the chloroform.

Normal butyl alcohol was first introduced for dealing with woody tissues, and is found to give excellent results when used on insect material. It has the advantage over alcohol of being both a solvent of paraffin wax and miscible with water; also the schedule is more rapid, while both shrinkage and hardening are considerably reduced. The schedule followed by the author is as follows:—

Step	1	2	3	4	5	6	7	8	9	10	11
Water	95	90	80	65	50	30	15	5	0	0	0
Ethyl alc.	5	10	20	35	40	50	50	40	25	0	0
N-butyl alc.	0	0	0	0	10	20	35	55	75	100	100
Hours	.5	.5	.5	1	1	1	12	1	1	1	..

The step at which dehydration is commenced varies with the fixative employed; if washing is necessary, step 1; after Kahle's fluid, step 4; and after Modified Kahle's, step 6. At step 10 pour the material and butyl alcohol into a vial containing an equal amount of solidified paraffin wax and place inside an oven; after about 16 hours change to pure paraffin wax, leave four hours and embed.

It has recently been pointed out that since 82 per cent butyl alcohol is saturated with water, material can be transferred to it directly from an aqueous medium. Moreover, because the vapour pressure of water is higher than that of butyl alcohol, material in 82 per cent butyl alcohol can be put directly into the oven, where the water is completely removed when from one-half to two-thirds of the liquid has evaporated. The writer has carried out these suggestions (although the material in question was fixed in Modified Kahle's fluid and thus was not completely aqueous) and finds the process entirely satisfactory.

For especially difficult subjects such as yolky, toughly chorionated eggs, which are prone to crumble in sectioning, the addition of 4 per cent phenol to steps 7, 10 and 11 is recommended. After embedding, the wax should be pared down to expose the embedded material in the required plane, and left soaking in water for at least 24 hours and preferably longer.

Dioxane, like butyl alcohol, is miscible with water and a solvent of paraffin. The schedules recommended are rapid and involve a minimum of changes: for example, wash in two or three changes of water; dehydrate in dioxane-water 1:3, 2:2, 3:1, leaving two hours in each; pure dioxane, 12 hours or overnight. Infiltrate wax of low melting point by adding small pieces while in the oven; four hours will usually be sufficient. Change to pure wax and leave two hours before embedding. It should be pointed out that dioxane in large quantities is sufficiently toxic to require keeping it as much as possible in a sealed vessel in a well ventilated room and using it at some distance from the nose.

iv) *Embedding*:

When the material has been placed in pure paraffin wax, smear an embedding dish with glycerin and place in the oven to reach the temperature of the

wax. In embedding, pour the liquid paraffin and material into the warm dish, add sufficient wax to ensure that the material is adequately covered (too much wax mitigates against rapid cooling), and by means of warm needles arrange the material in the centre of the dish. Carefully place the dish on the surface of a bowl of cold water and, while holding it here, blow on the surface of the wax until a film is formed which is sufficiently tough not to break when the dish is finally submerged. Rapid cooling is essential to prevent crystallization of the paraffin wax. When the wax is sufficiently cold the block should come out of the dish easily. Use hard wax (56° C) if thin sections are intended and soft (52° C) for thicker sections.

v) *Sectioning*:

Proficiency in the use of the microtome can be attained only after considerable practice; the general procedure is as follows:

a) trim the wax into a perfect rectangle viewed from the cutting surface with the material in the centre, leaving a moderate thickness around it; trim the cutting surface close to the material but leave about a quarter inch at the opposite side for attaching it to the mounting block.

b) arrange the mounting block in the holder so that the face of the wax is perpendicular and the whole lower edge of the wax will strike the microtome knife simultaneously, and cut at a uniform speed to ensure getting sections of uniform thickness. Try a thickness of 10 microns if the size of the chromosomes is unknown. If the sections tend to crumble as a result of using too soft a wax, flood the wax with alcohol and allow it to evaporate before continuing cutting; if the ribbon breaks due to the wax being too hard, place a lighted bunsen-burner near the knife or warm the stem of the mounting block.

c) transfer the ribbon to a sheet of glass placed over dark paper or onto a sheet of black ferro-type. Take chemically clean slides, mark them with the index number of the material, and smear with a thin coating of Mayer's albumen. Cut the ribbon into pieces about two inches in length and float them (shiny side down) onto the slides with water, taking care to maintain them serially so that the order of the sections is known.

d) place the slides on a warm plate or water-bath maintained at a temperature below the melting point of the wax and aid their stretching while arranging them side by side and parallel to the long edge of the slide; close attention to the neatness with which the sections are arranged will be amply repaid at the time of examination, especially if a mechanical stage is used on the microscope. Run off the excess water and leave to dry for at least three or four hours.

e) when completely dry dissolve off the paraffin by soaking for 5-10 minutes in xylol, rinse briefly in a second xylol and run down through a graded series of alcohols to water, or as far as 70 per cent if bleaching is necessary (see under smear method). If thick sections have been cut they may fail to adhere to the slide; use dried albumenized slides and float ribbons on with 35 per cent alcohol.

THE SMEAR METHOD.

The most satisfactory method of treating testes is by means of smearing, for a thin layer of cells is rapidly obtained, instantaneously fixed, and stained and differentiated with a minimum of trouble to preserve the finest details of meiosis. Its one limiting feature is that the relationship of the cells to each other is disturbed, rendering the seriation of the stages difficult.

Rapidity in fixation is the essence of good smearing, therefore prepare in advance the slide (marked with index number), a clean smearing scalpel (flat honed and preferably with a straight cutting edge) and a dish with parallel ridges about 2½ inches apart and containing enough fixative to cover the ridges. If ridged dishes are not available petri dishes with glass slips placed on the bottom will serve. Transfer the testes, dissected from the body cavity in frog

Ringer or saline, to the right-hand end of the slide. Place the scalpel, cutting-edge down, close to the right side of the testes, turn down onto the testes and, with rather more of the pressure exerted on the cutting edge, sweep rapidly and evenly across to the left-hand side of the slide, and immediately invert the slide in the fixative. After some minutes in the fixative remove any lumpy debris from the slide with forceps to ensure that when the coverslip is applied it will lie flat.

The most satisfactory fixatives are 2BD and CRAF. Fixation is completed in two hours and one will often suffice. Slides fixed in CRAF should be transferred straight to 70 per cent alcohol, but 2BD-fixed slides require 15 minutes washing in running water before running up through a series of alcohols (10, 30, 60 per cent, a minute in each) to 70 per cent. The blackening effect of osmic acid is bleached out by a mixture of 1 part H_2O_2 and 2 parts 80 per cent alcohol. Place the slide face down in the mixture and leave for 20-30 minutes in direct light; the slide is then ready to be run down to water prior to staining.

THE SQUASH METHOD.

This method has proved the most valuable of all for the study of insect chromosomes (see Plate I). By its use permanent slides can be obtained with a minimum of trouble in about two hours and the stain appears to have no tendency to fade. Almost any type of fixative will serve, but it has been found that many tend to harden the outer parts of the tissue before the unexposed parts are fixed, making it difficult to get a satisfactorily thin squash preparation. Further, since Feulgen is used as the stain, there appears to be no particular advantage in using a fixative containing chromic or picric acid. Satisfactory results have been obtained following the use of Carnoy's alcohol-acetic, but without doubt the best spreading and staining is obtained after Modified Kahle's fluid. There is, however, a possibility that further experimentation with chrom-osmic-acetic type fixatives may provide superior results on early stages at least.

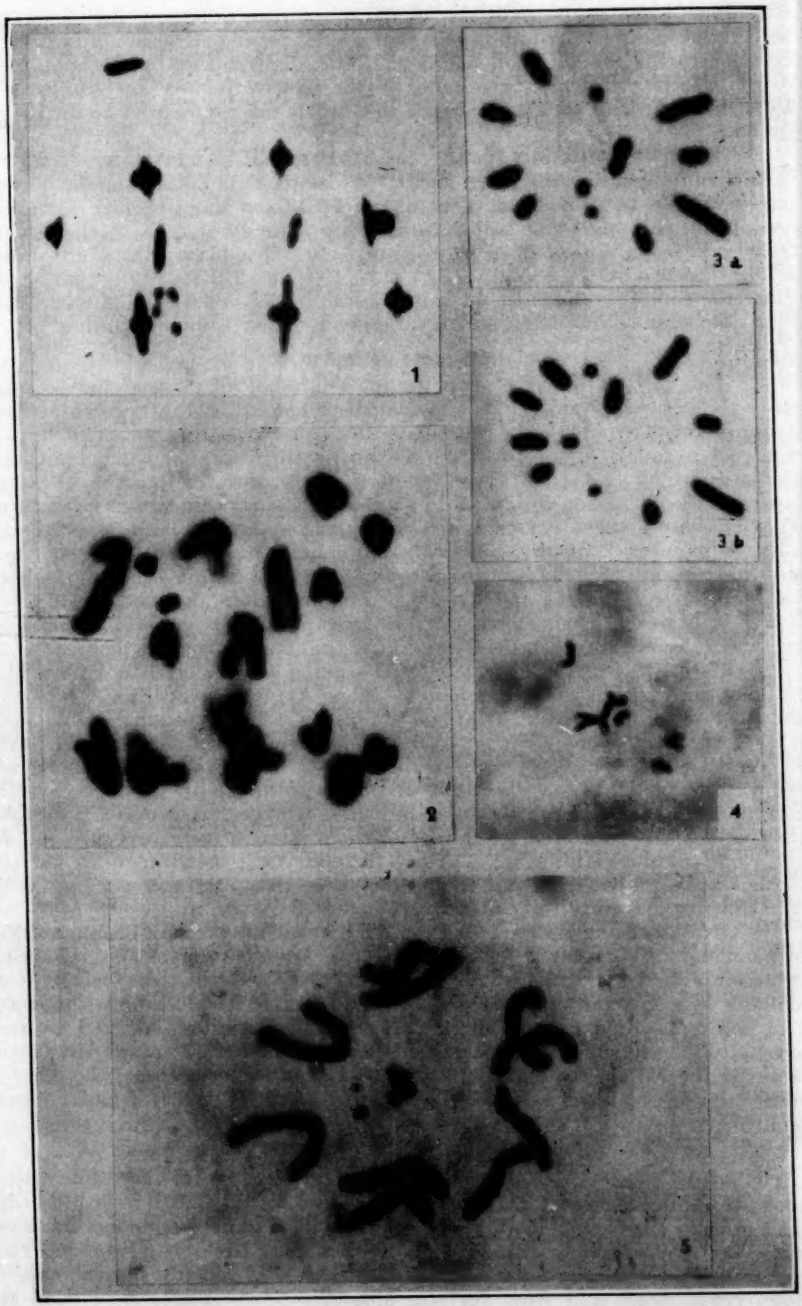
Fix for as little as 10 minutes and transfer the material to a drop of 45 per cent acetic acid on an albumenized slide. Apply a coverslip and heat as in the aceto-carmine method. Draw off the excess acid by pressure while under a layer of filter paper. Between successive heatings flatten the material further by applying considerable pressure to the coverslip by tapping and rubbing with the wooden handle of a dissecting needle or rolling with a squeegee roller. Examination under the microscope will disclose whether the material is squashed sufficiently thinly and also, if the chromosomes are relatively large, the number and stages of the dividing cells. Place the slide in a flat dish of 70 per cent alcohol and, steadying one end of the coverslip with the finger tips, pry it off. The slide may remain in the alcohol until ready for staining.

Occasionally it is found that material which has already been embedded in paraffin wax is required for examination in squash preparations. This can be accomplished by paring off as much wax as possible and dissolving the remainder in a mixture of equal parts of xylol and butyl alcohol on the top of a heated embedding oven. Three changes of the mixture followed by pure butyl alcohol will suffice, after which the material should be transferred first to 70 per cent ethyl alcohol and then to 45 per cent acetic acid at 60° C. After 15 or 20 minutes the material is sufficiently softened to give a flat squash preparation.

II. STAINING METHODS.

The four stains most commonly used are carmine, crystal violet, haematoxylin, and basic fuchsin. Whenever possible it is advisable to avoid using haematoxylin as a simple chromosome stain, as its opaqueness renders the interpretation of overlapping chromosomes difficult. It has the advantage, however, of staining the centrosomes and spindle, neither of which is normally visible after crystal violet and basic fuchsin, although aceto-carmine may stain the spindle on occasions.

PLATE I.



TECHNIQUES FOR THE STUDY OF INSECT CHROMOSOMES

CRYSTAL VIOLET STAINING.

There are many advantages to be gained from using crystal violet; the material is in water for a minimum of time, the procedure is rapid and simple to use, the cytoplasm is colourless, thus giving the maximum of differentiation, and sections from 4-40 microns in thickness can be stained with ease. This last fact, together with the transparency of the stain, is perhaps its greatest asset. It is not unlikely that many of the erroneous chromosome counts reported in past years can be attributed to the use of haematoxylin; its opaqueness, and the difficulty encountered in differentiating thick sections, led to the use of sections which had been cut too thin, with the result that many of the plates were incomplete. The only disadvantage of crystal violet is its tendency to fade, although this is less marked than with the older uncertified gentian violets which appear to be mixtures with methyl violet. The main reason for the fading of certified crystal violet appears to be the use of balsam having an acid reaction; care should be taken to secure neutral balsam and to dilute it, when necessary, only with non-acidified xylol. If neutral balsam is unavailable a small piece of NaOH may be kept in the jar, or one of the synthetic media such as "Nevillite" can be used.

The standard procedure for the crystal violet method is as follows:

Stain 3—10 minutes in 1 per cent aqueous crystal violet, boiled, cooled and filtered.
Rinse in tap water.
Mordant in iodine (1 per cent iodine + 1 per cent KI in 80 per cent alcohol) 30-45 seconds.
Rinse in 95 per cent alcohol.
Wash in absolute alcohol 2—10 seconds.
Differentiate in clove oil under the microscope.
Clear in xylol, 3 or 4 changes, for at least 5 minutes and preferably 30 or more in the last.
Mount in Canada balsam.

With certain insects, especially those having small chromosomes, it is often difficult to get the crystal violet to remain in the chromosomes, while removing it completely from the cytoplasm. This may sometimes be remedied by partial differentiation before mordanting in iodine; immediately after washing off the excess stain rinse briefly (1-5 seconds) in 95 per cent alcohol, taking care not to remove the stain entirely. Since alcohol removes the stain from the cytoplasm more readily than from the chromosomes, less stain remains in it to be precipitated by the iodine. However, following fixatives devoid of chromic acid, it is usually impossible to obtain successful staining by either the ordinary method or this modification. This can often be overcome by mordanting in a 1 per cent solution of chromic acid for 12 hours or overnight, followed by washing in running water for about 30 minutes before staining. In more obstinate cases the subsequent staining should be modified as follows:

Stain 10-30 minutes in 1 per cent aqueous crystal violet.
Rinse in tap water.
Absolute alcohol 2-3 seconds.
Iodine 1½-2 minutes.
Absolute alcohol 2-3 seconds.
Chromic acid, 1 per cent, 15 seconds.
Absolute alcohol 5 seconds.
Chromic acid 15 seconds.
Absolute alcohol 5-15 seconds.
Differentiate in clove oil.
Clear in xylol.

The mordanting in iodine may precede the staining, and in combination with picric acid in absolute alcohol gives an intense almost black colour to the chromosomes against a very pale yellow cytoplasm:

Mordant in iodine 10-20 minutes.
Rinse in water.
Stain 5-20 minutes.

Rinse in water, then iodine, then 95 per cent alcohol.
Wash quickly in absolute alcohol saturated with picric acid.
Wash in pure absolute alcohol a few seconds.
Differentiate in clove oil.
Clear in xylol.

The excellent differentiation between the chromosomes and the cytoplasm makes this process especially favourable for the examination of prophase stages, and is said to give greater permanency.

Mordanting in iodine is sometimes completely eliminated, its place being taken by picric acid added as crystals to a series of alcohols (10, 30, 70, 83 and 95 per cent) to make an 0.5 per cent solution. Leave in each for about 15 seconds; then 95 per cent alcohol with 3 drops of NaOH added to each 100 cc; absolute alcohol; clove oil; and xylol. The series should be renewed after approximately 60 slides. This procedure allows greater latitude of time spent in clove oil and hence better differentiation is possible.

Although, in the case of insects, it is usually difficult to secure a stain of sufficient intensity, it sometimes happens that the stain is difficult to remove from the cytoplasm and the chromosomes. This can be alleviated to a considerable extent by thinning the clove oil with an equal amount of absolute alcohol. However, as staining is better when differentiation is short, the time in the stain should be adjusted, and the concentration of the stain lowered, so that differentiation is as rapid as possible.

HAEMATOXYLIN STAINING.

Despite its many drawbacks haematoxylin can at least be relied upon to give permanent staining, and it is invaluable in the study of chromosome mechanics as a result of its selective staining of the spindle and centrosomes. The two commonest procedures in using haematoxylin are those of Delafield and Heidenhain; they differ primarily in that mordanting and staining are carried out as two separate operations in the latter and combined in the former.

i) *Delafield's haematoxylin*: To 400 cc. of a saturated aqueous solution of ammonia-alum add 4 gm. of haematoxylin crystals dissolved in 25 cc. of 95 per cent alcohol. Ripen by exposing to light and air for 3-4 days, or oxidize rapidly by adding a small quantity of H_2O_2. Add 10 cc. of glycerin and 100 cc. of methyl alcohol and filter; the solution keeps indefinitely. Stain 20-30 minutes or longer; differentiate in alcohol.

Sectioned eggs are particularly difficult to stain owing to the abundance of yolk which, because of its strong affinity for haematoxylin, frequently obliterates the chromosomes. The following modification of Delafield's formula is valuable in overcoming this: to 10 cc. of Delafield's haematoxylin and 40 cc. of distilled water add 10 drops of picro-sulphuric acid (a saturated solution of picric acid in 2 per cent sulphuric acid). Stain 5-10 minutes and wash in alcohol. The presence of the acid prevents the yolk from staining and allows the haematoxylin to act on the chromosomes alone.

ii) *Heidenhain's haematoxylin*: For mordanting add to 100 cc. of distilled water 4 gm. of iron-alum crystals (ammonio-ferric sulphate) which have been carefully selected so that all are a pure violet colour and not oxidized. For staining dissolve ½ gm. of haematoxylin crystals in a small quantity of 95 per cent alcohol and dilute to a 0.5 per cent aqueous solution.

The classical procedure is as follows:

Mordant in 4 per cent iron-alum 12 hours or overnight.
Wash 5-15 minutes in running water.
Stain in 0.5 per cent haematoxylin for a period of time equal to that of mordanting.
Rinse in water.
Differentiate in 2 per cent iron-alum under the microscope.
Wash thoroughly in water 15 minutes or longer.
Dehydrate, clear in xylol, mount in balsam.

The length of mordanting, washing, staining and most particularly the age of the solutions have some effect on the final result; fresh haematoxylin in particular giving a bright blue stain while older solutions range towards black.

A saturated aqueous solution of picric acid can be substituted for iron-alum in differentiation; it produces a more transparent stain, leaves a cleaner cytoplasm and hence gives a sharper definition, and due to its milder destaining action allows a more precise picture to be obtained.

In recent years there has been a decided tendency to shorten the times of mordanting and staining, especially in combination with more concentrated solutions of both. For both smears and sections 30 minutes in 4 per cent iron-alum, followed by 10-15 minutes washing and 60 minutes in 2 per cent haematoxylin provides most satisfactory results. Differentiation, however, should be carried out under the microscope in a solution of iron-alum as weak as 0.5 per cent until rather more stain has been removed than appears advisable. Washing for at least 15 minutes and dehydration followed by xylol will clear the cytoplasm sufficiently to restore the maximum contrast between it and the chromosomes.

The most rapid schedule consists in carrying out the mordanting and staining at a temperature of approximately 55° C. Place the slides soaked in water on a hot plate; flood with 4 per cent iron-alum and allow to steam, adding more as necessary; continue for several minutes; rinse in water and replace with 0.5 per cent haematoxylin for several minutes; return to water and differentiate in cold 2 per cent iron-alum; wash 5 minutes, or preferably longer.

iii) *Brazilin*: This natural dye, obtained from Brazil wood, has general properties similar to haematoxylin, but is weaker and less active and gives a reddish-brown colouration instead of blue-black. According to Belling (1) it is used in alcoholic solution in the following manner:

Mordant in 4 per cent iron-alum in 70 per cent alcohol for 24 hours.
Wash briefly in 70 per cent alcohol and soak for 15 minutes to 3 hours in 70 per cent alcohol.
Stain in 0.5 per cent brazilin in 70 per cent alcohol (to which has been added 1 or 2 drops of the iron-alum to each 50 cc.) for 2-24 hours.
Wash briefly in 70 per cent alcohol.
Differentiate in 1 per cent alcoholic iron-alum for from 1 minute to 3 hours or more.
Wash in 70 per cent alcohol and dehydrate to absolute alcohol.
Clear in alcohol-cedar oil and mount in immersion cedar oil.

The intensity of the stain both in the chromosomes and the cytoplasm varies with the amount of washing out of the mordant, the shorter the washing the deeper the stain. Further, prophase stages require only short staining and slight differentiation, while later stages and small chromosomes need longer in both the stain and the differentiating fluid. The method is especially valuable for smears, since prior to staining these are less hardened than sectioned material and the maceration consequent upon the use of aqueous haematoxylin is largely avoided.

THE FEULGEN STAIN.

The red dye, basic fuchsin, is decolourized by sulphur dioxide; the colour of this reagent (known as Schiff's reagent) is promptly restored by a few drops of an aldehyde solution. When thymonucleic acid is subjected to mild acid hydrolysis, aldehyde groups are liberated which react with leuco-basic fuchsin (a solution of pararosanilin, decolourized by the action of sulphur dioxide) to give a purplish colour. This adaption, developed in 1924 by Feulgen and Rossenbeck, is the basis of the most specific of all nuclear stains; Feulgen's "nuclealfärbung".

Even after it was shown that not only the sublimate-acetic fixative recommended by Feulgen but all common fixatives could be used before the Feulgen stain, the method found only restricted use among cytologists. Many authors refer to a "negative" Feulgen reaction for which two main reasons are respon-

sible. First, there are types of nuclei, such as the growing oocytes of insects, in which the chromatin is very dispersed and may be easily overlooked. Second, the technique employed was often inappropriate, either because of an unsatisfactory stain or the incorrect time of hydrolysis. Many commercial American stains failed to give a positive reaction due to the presence of impurities, methods for the removal of which have now been worked out. It has been shown that the optimal hydrolysis time varies with the fixative used.

Solutions needed for the Feulgen stain:

(1) Normal HCl: Hydrochloric acid (spec. grav. 1.19) 82.5 cc.
Distilled water 917.5 cc.

(2) Leuco-basic fuchsin stain: pour 200 cc. boiling distilled water over 1 gm. pararosanilin and shake. Cool to 50° C, filter into brown stock bottle with ground-glass stopper and add 20 cc. N HCl. Cool to room temperature and add 1 gm. anhydrous sodium bisulphite ($NaHSO_3$). Keep stain in the dark. After 24 hours the solution should be colourless or a light straw colour. If it does not become colourless or later turns reddish, it is useless but can be decolourized by adding more $NaHSO_3$.

Potassium metabisulphite ($K_2S_2O_5$) is a better decolourizing agent than $NaHSO_3$, and is used in the same or double the quantity of the latter. If the staining solution does not decolourize properly, add 0.5 gm. of powdered carbon, shake thoroughly and filter immediately.

(3) Sulphite washing fluid: Water 100 cc.
10 per cent aqueous $NaHSO_3$, or $K_2S_2O_5$ 5 cc.
N HCl 5 cc.

Make up immediately before use.

Use of the Feulgen stain: Transfer the slide from water to cold N HCl and rinse. Hydrolize in N HCl at 60° C for 5-16 minutes. Rinse in distilled water. Stain in leuco-basic fuchsin for ½–1½ hours. Drain and pass quickly into the first of three coplin jars containing the sulphite washing fluid. Leave in each for 5 minutes. Rinse in tap water. Run up through a graded series of alcohols to absolute alcohol, two changes of xylol and and mount in balsam. As alternative dehydrating series either the alcohol-acetic mixtures used after aceto-carmine or the dioxane-water mixtures used in embedding may be employed; there do not appear to be any special advantages in their use however.

It has been found that the optimal hydrolysis times vary with the fixative and, using agar blocks impregnated with nucleic acid, B. B. Hillary has found that this variation is due to differences in the chromic acid content of the fixative. With fixatives free from chromic acid the maximal stain is produced following 4-8 minutes hydrolysis; with prolonged treatment there is a gradual falling off of the stain until at 20 minutes no further stain is visible. On the other hand, the optimal staining curve for fixatives containing chromic acid shows a flat-topped maximum from 5-30 minutes. Hence 5 minutes hydrolysis after any of the common fixatives is recommended. With insects, however, it has been found that differences inherent in the material are often greater than variations introduced by the fixatives; the general rule may nevertheless be applied once the optimum hydrolysis time for a specified fixative is determined.

Most of the common counterstains can be used in combination with Feulgen. It is usually recommended that animal tissues be treated for 3-5 minutes in a 1 per cent aqueous solution of orange G; in practice a 0.5 per cent solution of light green in 70 per cent alcohol will be found less tiring to the eyes as it tones down the colour of the Feulgen stain, and also stains the nucleoli green thus locating the nucleolar-attachment chromosome. The counter-staining is done in the course of dehydrating; stain 1 minute, wash off the excess dye, differentiate in 70 per cent alcohol, and complete the dehydration in the usual manner.

The Feulgen stain can be applied after other methods, tried on the same slide, have proved unsuccessful, but the reverse is not always true. If the Feulgen reaction fails due to excessive hydrolysis, aceto-carmine will usually succeed, but crystal violet staining is often weak or impossible.

To remove coverslips prior to restaining, simply soak in xylol until the coverslip separates from the slide. A more rapid method consists in soaking for a few hours in a mixture of xylol and normal butyl-alcohol, 9 parts to 1; this solution will, however, wash out anilin dyes.

Feulgen staining of entire eggs: Due to the fact that the Feulgen stain does not colour the yolk, it is especially valuable for the study of meiosis in the eggs of insects. The use of sectioned material for this purpose is extremely tedious, as a great many sections must be examined when searching for the relatively minute nuclei. The method of preparing entire eggs consists of fixing them in a modification of Carnoy's fluid (equal parts of chloroform, absolute alcohol and glacial acetic acid) for ½-2 hours, washing in absolute alcohol for two hours and running down to water. If the eggs are particularly small, they should be packed in *Drosophila* pupal skins to facilitate handling. Stain according to the Feulgen schedule, dehydrate rapidly, clear in xylol and mount in balsam. The eggs can be rotated into the appropriate position by moving the coverslip. This is best done soon after the coverslip is applied, but if necessary movement of the coverslip is possible for some time after mounting if the hardened edges of the balsam are sottened with xylol. It is self-evident that ample balsam must be used to ensure that the coverslip does not press down on the eggs.

A more rapid technique consists of isolating living eggs in a very small drop of water on a slide, pricking the membrane of each with a fine needle and allowing the contents to flow out while dragging the membrane away. The contents of the egg spread and dry so quickly that the chromosomes are 'fixed' immediately. The original (1905) preparations made by this method from annelids and stained with either iron-haematoxylin or aqueous Bismark Brown have provided results which compare favourably with those obtained by any other procedure. The author has found this method, combined with Feulgen staining, to surpass all others when used on *Drosophila* eggs.

STAINING OF SALIVARY GLAND CHROMOSOMES OF DIPTERA.

No paper on the techniques used in the study of insect chromosomes would be complete without some mention of the special methods used on salivary gland chromosomes. Basically the procedures are those already given for the ordinary aceto-carmine and Feulgen-squash methods, but certain refinements in both are necessary in order to obtain the best results. The following description applies specifically to *Drosophila*, but will serve as a working basis for all Diptera.

i) *Temporary Aceto-carmine Preparations*: To obtain a stain of sufficient intensity, boil the carmine in 45 per cent acetic acid for several hours with a reflux condenser attached to prevent evaporation. No iron should be added for intensification as this prevents stretching of the chromosomes. Dissect the glands in ice-cold Ringer (cold-blooded formula) or 0.73 per cent NaCl, and stain in ice-cold aceto-carmine in deep depression slides for not longer than 15-20 minutes, otherwise the chromosomes become fragile and cannot be stretched Using silvered needles, transfer to albumenized slides free from dust and lint, wash in fresh aceto-carmine, and remove any attached fat bodies or other debris. Apply coverslip and remove excess stain with filter paper. Spread the chromosomes with controlled pressure while under the dissecting microscope, taking care not to move the coverslip, as this will cause the cells to roll up into dense useless masses. "Ripen" the preparations by storing in the refrigerator for two to three days; replace the aceto-carmine by 45 per cent acetic acid and seal with gum mastic-paraffin.

ii) *Permanent Aceto-carmine Preparations*: To make temporary slides permanent, "ripen" them by storing over night in saturated 95 per cent alcohol vapour in ground-glass stoppered bottles lined with filter paper soaked in alcohol. This "vapour method" of replacing aceto-carmine by alcohol allows dehydration with a minimum of shrinkage. Immerse in 95 per cent alcohol; the coverslip may detach itself or can be pried off while lying flat in a petri dish of alcohol. Drain the slide and mount rapidly in thin Euparal or "Nevillite", removing excess by blotting.

iii) *Feulgen Preparations*: The procedure up to the removal of the coverslip is the same as above, using either aceto-carmine or 45 per cent acetic acid. After removal of the coverslip run down through a graded series of alcohols to water and stain according to the Feulgen schedule. Post-fixation in 3 parts 1 per cent chromic acid and 1 part of formaldehyde for 10-12 hours is sometimes advocated before hot HCl; follow with 25 minutes hydrolysis). Dehydrate to 95 per cent alcohol and mount as above.

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EXPLANATION OF PLATE I.

Photomicrographs taken from slides prepared by the squash method (Modified Kahle's fluid and Feulgen stain) and reproduced at a magnification of 2750x, except for Fig. 1 which is only 1830x.

Figs. 1, 2 and 3a and b. Spermatogenesis in *Melanoplus femur-rubrum* (DeG.): Fig. 1, the complete complement (11 bivalents and, above, the univalent sex chromosome) at the first meiotic metaphase; Fig. 2, the first anaphase with the sex chromosome, the fourth from the right, going undivided to the upper pole; Fig 3a and b, two second anaphase complements containing 11 autosomes and 1 sex chromosome each. Note the similar spatial arrangement.

Fig. 4, the seven somatic chromosomes from the brain of the male of *Neodiprion lecontei* (Fitch) (haploid)

Fig. 5, somatic pairing of homologous chromosomes from the mesenteron of *Bessa selecta* (?). Note the close association especially at the constricted regions (except where forced apart in squashing).

NOTES ON THE *BETULELLA* GROUP OF *DEPRESSARIA*, WITH DESCRIPTION OF NEW SPECIES. (LEPIDOPTERA, OECOPHORIDAE) *

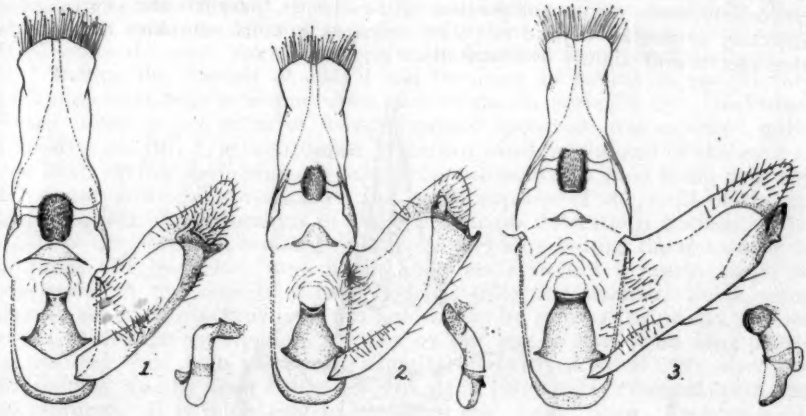
BY J. McDUNNOUGH
Ottawa, Ont.

In his recent Revision of the Oecophoridae (1941, Proc. U. S. N. M. Vol. 90, No. 3107) Gates Clarke (p. 196) records the larva of *Depressaria betulella* Bsk. as feeding on both *Betula* and *Ostrya*. As material bred by us from *Ostrya* this past summer in the Ottawa region did not at all match with our series of the birch-feeder I have made a genitalic study of the species of this group in the Canadian National Collection in order to check up on the matter.

Both from bred material and according to Clarke's key to the adults our identification of *betulella* seems correct; the primaries are a pale fawn with a short black streak along the base of the inner margin and with the black discal dash between the pale spots practically lacking. Slides of the female genitalia match well with Clarke's fig. 276 but it would seem that some error has been made in his fig. 193 of the male genitalia; this does not represent the birch-

*Contribution No. 2204, Division of Entomology, Science Service, Department of Agriculture, Ottawa.

feeder at all but does match excellently with my slide of the male *Ostrya*-feeder. The male genitalia of the true *betulella*, as exemplified by several slides made from both bred and collected material, are much closer to those of *grotella* than are those of the *Ostrya*-feeder. The clasper (harpe of Gates Clarke) is, however, narrower and more pointed apically; the harpe (clasper of Gates Clarke) is of the same general bifid type, extending transversely across the apical portion of the clasper; in width it is narrower than that of *grotella* and shows other minor differences in the shape of the two prongs. I do not believe that the shape of either the transtilla or the juxta can be used satisfactorily for separation, as there seems to be considerable individual variation in these portions, due probably to their very weak chitinization; the gnathos is noticeably larger in *betulella* and this may prove a more stable character; the aedeagus is very definitely distinct, being sharply elbowed apically with a small projection or tooth on the upper side, near apex. These above-mentioned differences can be best



Male Genitalia of 1. *Depressaria betulella* Bsk.; 2. *D. grotella* Rob. 3. *D. ostryella* n. sp.

visualized by a comparison of my figure of *betulella* (fig. 1) with that of *grotella* (fig. 2) which appears to agree with Gates Clarke's figure in all essential details. Strangely enough in his "Key to Species", based on male genitalia (p. 167) the captions separating *betulella* and *grotella* are correct.

Grotella Rob. seems to have been definitetly tied down to the hazel-feeder. The original description and colored figure leave much to be desired from the standpoint of accurate identification but I presume that Gates Clarke has been able to study the type in the American Museum, although he does not say so. In any case, as already indicated, my slides of the genitalia of this hazel-feeder correspond with the illustrations given in the Revision of *grotella* and I am, therefore, employing the name in a similar manner. The larvae ball-up the young leaves of hazel in a quite characteristic manner, they differ from those of *betulella* in lacking the prominent bases of the abdominal setae, being a unicolorous pale green with light flesh-colored head and black-marked ocelli.

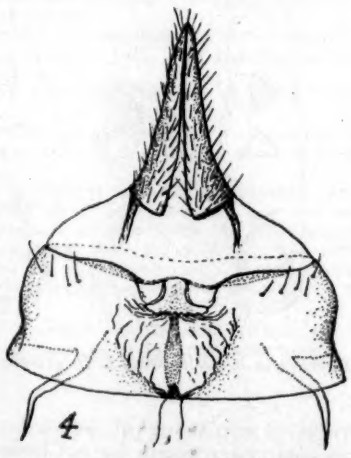
The *Ostrya*-feeder is very similar in general appearance to *grotella*, having the primaries of the same shade of brown (light mahogany-brown) and, in consequence, appearing much darker than *betulella*. The larvae live between folded leaves and are quite similar to those of *grotella*, showing, however, considerable dark shading on the sides of the prothoracic shield, a character apparently lacking in the allied species. As the genitalia in both sexes show definite

distinctions from those of both *betulella* and *grotella* I am treating the species as new and describe as follows:

Depressaria ostryella n. sp.

Palpi ochreous, second joint lightly sprinkled on outer side with smoky, third joint with apical half blackish. Head ochreous. Thorax and patagia much deeper in color, brown with slight ochreous suffusion. Primaries similar in color and maculation to those of *grotella* and hardly to be distinguished from same. There is perhaps rather more light ochreous shading along the basal portion of costa and the dark discal dash between the pale spots is possibly somewhat less prominent; the marginal row of black dots seems also more evanescent but these differences are scarcely sufficient to render a definite identification possible and resort must be had to the genitalia in doubtful cases.

Male Genitalia. Of the same general type as that of other members of the group. The main distinction lies in the harpe which, instead of running transversely across the apical portion of the clasper, parallels the ventral edge, projecting somewhat beyond it. The aedeagus is much chunkier than in the other species and slightly bent apically.



4. Female Genitalia of *Depressaria ostryella* n. sp. (Allotype).

Female Genitalia. Ovipositor much more pointed and with more closely appressed lobes than in either *grotella* or *betulella*. Genital plate much broader than long, with a large central semicircular, irregularly crinkled mound, resting on the cephalic margin of the plate. The ostium is situated at the base of a broad central excavation of the caudal margin of the plate, the rear margin of the aforesaid mound forming a partial protection to same. The initial portion of the ductus bursae is narrowly spindle-shaped and weakly chitinized; following this is a short straight membranous section, beyond which the ductus forms the numerous convolutions characteristic of the group. Bursa copulatrix a large sac with well-developed signum.

Holotype—♂, S. March, Ont., June 17, 1942 (J. McDunnough), (bred on *Ostrya*) No. 5388 in Canadian National Collection.

Allotype—♀, Merivale, Ont., June 21, 1942 (J. McDunnough), (on *Ostrya*).

Paratypes—1 ♀, same data as Allotype; 1 ♀, Kirksferry, Que., June 24, 1942 (Dom. Forest Ins. Survey) (On Ironwood).

The above localities are all in the Ottawa region.

NOTES ON THE DISTRIBUTION OF *PLEBEIUS SCUDDERI EMPETRI* FRMN. AND *P. SCUDDERI ASTER* EDW. (LEPIDOPTERA, LYCAENIDAE) *

BY T. N. FREEMAN
Ottawa, Ont.

Plebeius scudderi empetri Frmn. was described from material taken in early July, 1936, in an *Empetrum* bog not far from the town of Baddeck on Cape Breton Island. Later in July of that year several similar bogs were observed in various parts of Nova Scotia, but with the exception of a single worn specimen captured in the Cape Canso region, no specimens of *empteri* were observed. This was thought to be due to the lateness of the season.

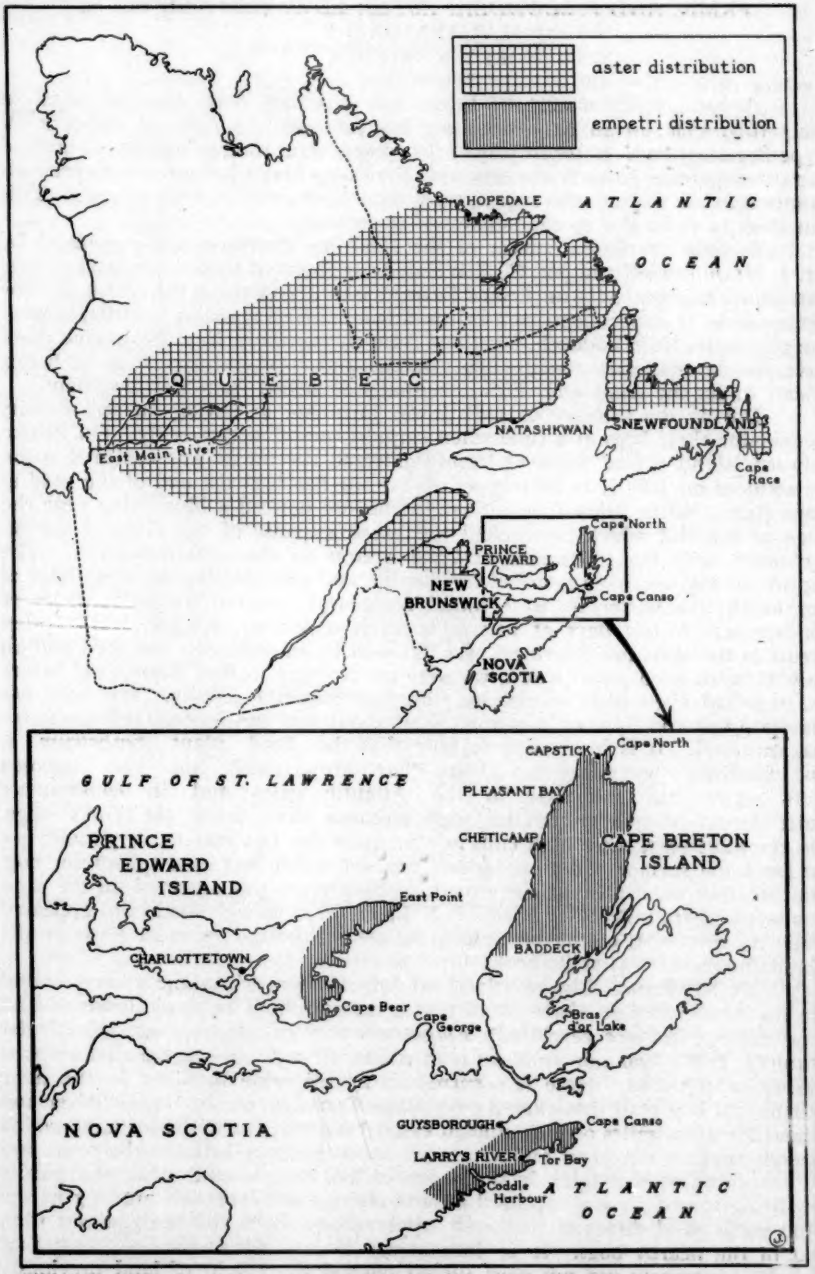
In 1940 the faunal survey of the Maritime Provinces was continued by Dr. J. McDunnough and Mr. G. S. Walley who observed similar *Empetrum* bogs containing this butterfly on Prince Edward Island and the north shore of New Brunswick. The specimens from Prince Edward Island agree in maculation with the type series from Baddeck, but those from the northern New Brunswick shore have, for the most part, smaller spots on the underside and appear to be better placed under the name *aster* Edw., originally described from Newfoundland.

During the summer of 1941 I was fortunate in having an opportunity to examine these bogs at a time when *empetri* should normally fly. On Prince Edward Island a fine series of freshly emerged specimens was captured quite by accident on July 7, as we stopped to eat our lunch at the end of the road at Cape Bear. While munching our sandwiches we noticed a blue flying near the edge of the cliff which overlooked the broad expanse of the Gulf of the St. Lawrence, with the mountains of Cape Breton on the eastern horizon. The capture of this specimen revealed its identity, and after bolting the remainder of our lunch, the butterflies were found abundantly around a nearby patch of *Empetrum*. A few days of utterly hopeless collecting weather, which often occurs in the Maritime Provinces, was followed by an unusually fine day, and on July 11, with keen anticipation, we were on our way to East Point long before the sun had risen high enough to stimulate butterfly activity. We were not disappointed, and by noon a nice series of about fifty freshly emerged specimens was captured. It is interesting to note that the food plant *Empetrum* is not confined to sphagnum bogs, but grows well on the exposed cliffs which are subjected to the Atlantic gales and in consequence quite devoid of tree growth for some distance back from the cliff's edge. For the most part, these high cliffs appear quite dry but may be very moist not far from the surface and thus imitate the wet conditions of a sphagnum bog. On the other hand a luxuriant growth of *Empetrum* was observed on the sand dunes not far from Brackley Beach, P. E. I., and even though conditions appeared ideal, no specimens of the butterfly were seen, although on other parts of the island emergence was at its peak.

We left Prince Edward Island on July 15 to continue the survey around the northeastern coast of the Nova Scotia mainland. The scenic drive around Cape George did not reveal any *Empetrum* nor, in consequence, any of the butterfly, and it was not until we reached the Cape Canso region that *empetri* was again captured. Here it occurred in unbelievable numbers in the huge expanses of bog land that extend from Cape Canso proper to Larry's River and inland for a few miles on the exposed eastern slope of the height of land, which extends roughly northeast and southwest about halfway between the coast and the town of Guysborough. In the region of Tor Bay, a small series of typically low flying specimens was captured around clumps of *Empetrum* growing in the wind swept sand dunes of that area, apparently quite a different habitat than that of the nearby bogs. It is interesting, if not significant, to note that *empetri* apparently did not exist inland beyond the height of land previously

*Contribution No. 2192, Division of Entomology, Science Service, Department of Agriculture, Ottawa.

PLATE II.



DISTRIBUTION OF *PLEBEIUS SCUDDERI EMPETRI* FRMN. AND OF *P. SCUDDERI ASTER* EDW.

referred to, although the food plant grows well there on some of the exposed hilltops. This condition led me to believe that the insect apparently preferred those points and capes which were facing the east and exposed to the cold winds of the Atlantic. In the Canso region, series were captured at Cape Canso proper, Tor Bay, Lundy and Coddle Harbor.

As the *empetri* season was well advanced in the Canso region by July 21, we continued our survey north and east to the ancient hills of Cape Breton. With headquarters temporarily established at Baddeck, we proceeded north on the Cabot Trail to the village of Cape North where we left the Trail and continued on a secondary road toward Cape St. Lawrence and Cape North. The little villages of Meat Cove and Capstick mark the end of the road and more or less the end of the English language, for here the Celtic descendants usually speak Gaelic and accent the English 's' with a decided hiss. This brings to mind one Archie McInnis of this district who is anxious to receive a mineral detector for the purpose of locating an ancient pirate treasure of untold wealth, marked on the rock underwater by a cross, and pointed out to Archie by his grandfather when the former was a lad. Unfortunately, the exact position of the cross has been forgotten and diligent search has not revealed it. Here, as well, the *Empetrum* grows on the high, apparently dry cliffs of the most rugged coast-line I have ever seen, where one lone male *empetri* was captured, marking the northernmost point of its known distribution.

Leaving the town of Cape North and proceeding toward Pleasant Bay, we observed a huge sphagnum bog on the top of the height of land which extends parallel to the western coast-line and not far from the latter. *Empetrum* abounded here, and a fine series of the blue was captured on July 24. A similar bog exists on the mountains above Cheticamp to the south and on the same date *empetri* was abundant at this locality, although the specimens were badly rubbed. It is quite probable that these *Empetrum* bogs exist on the flat tops of many of the mountains in the interior of Cape Breton and support an abundant population of the blue.

From this survey I limit the known distribution of *empetri* to that part of the Canadian Life Zone occupying the east coast of Prince Edward Island, the northeastern part of the mainland of Nova Scotia and that section of Cape Breton lying northwest of Bras d'Or Lake.

Aster Edw. (1882, Can. Ent., XIV, 194) was described from one male and one female, part of a considerable series, taken by Mr. T. L. Mead in the Cape Race region of southern Newfoundland. Newfoundland appears to consist of three life zones, the Arctic, Hudsonian, and Canadian, and I consider that the Cape Race region lies within the Hudsonian zone. *Aster* appears to restrict itself to this latter life zone, and an examination of specimens from other parts of this zone appears to support this restriction. In intermediate localities between these two zones, intergrades of *aster* and *empetri* occur, and unless a series from any such locality is examined, it is usually difficult to apply a name. For example, the northern New Brunswick specimens, previously referred to, exist in an intermediate locality and intergradation is evident. Such a condition appears to exist in the Codroy Valley region of southeastern Newfoundland as represented by two specimens before me which possess somewhat larger spots than typical *aster* and would appear better placed under *empetri*. As this locality is represented on maps as occurring in the Canadian Zone, such a condition is understandable.

From an examination of specimens before me, I consider the known distribution of *aster* to occupy that portion of the Hudsonian Zone which includes the north and eastern parts of Newfoundland, northeast to Hopedale, Labrador, south through Natashkwan, Que., to the northern shore of New Brunswick and west to the East Main River region of James Bay. The accompanying diagram roughly outlines the known distribution of these two races of a complex and somewhat variable species.

NOTES

BLACK FLOUR BEETLE, *TRIBOLIUM MADENS* CHARP., IN BRITISH COLUMBIA (COLEOPTERA, TENEBRIONIDAE)

M. H. Hatch, quoting Cotton and Good, 1937, has reported *T. madens* as occurring under bark or in decaying tree trunks in eastern North America. (1942. The biology of stored grain insects. Bull. Assoc. Operative Millers (for July), pp. 1207-1211).

This same species (det. F. E. Blaisdell) is common in the southern interior of British Columbia. At Salmon Arm my father found many of the beetles inside the tops of his beehives on April 3, 1932, and later they were common under boards and stones nearby. Earlier the same spring, I had found adults of *madens* actually attacking and killing the almost immobile larvae of Bibionidae in leaf mold under a log in the woods at Salmon Arm.

The following additional British Columbia localities are from specimens in the collection of the late Ralph Hopping: Aspen Grove, 15. VI. 33; Fernie, 8. VI. 34; Mara, 1921; Midday Valley, Merritt, 11. VII. 23, under bark *Pinus ponderosa;* Quesnel, 10. VII. 32; Trinity Valley 24. V. 25, under bark *Pinus monticola;* Vernon, 15. VII. 31, in wheat; Waterloo Mine, August, 1934.

Hugh B. Leech, Vernon, B. C.

NOTE ON *MARMARA FASCIELLA* CHAMBERS

The food plants of all North American species of *Marmara* save *M. leptoderma* Meyr. and *M. fasciella* Cham. are known. It has now been found that *M. fasciella* makes a long, linear mine in the bast of the trunk and branches of Eastern white pine (*Pinus strobus*). The cocoon is spun in the end of the mine under a loosened flap of the bark; it is not ornamented with globules. Adult emergence takes place from the last days of May until the first week in July. Eggs are laid on the bark and the hatching larvae penetrate into the bast through the underside of the eggs. About one-half of the mine is constructed during late summer and fall. The larvae hibernate inside of the mine and resume their feeding early in the spring of the following year. The mines are usually completed between May 15 and June 15.

The species is probably distributed over the entire range of Eastern white pine. Mines and larvae, apparently similar in every respect to those of *M. fasciella*, have been found in balsam fir (*Abies balsamea*) in Ontario, and in Western white pine (*Pinus monticola*) in British Columbia. No adults were reared from this material.

M. fasciella is heavily parasitized by the following Hymenoptera: *Copidosoma* sp., *Habrocytus* sp., *Hemiteles tenellus* Say, *Pleurotropis* sp. (near *niger* Ashm.), *Pleurotropis* sp. (probably *lithocolletidis* Ashm.), *Elachertus* sp.

The parasites were determined by Dr. O. Peck, of the Division of Entomology, Ottawa. My determination of *M. fasciella* was confirmed by Mr. Carl Heinrich, of the U. S. Bureau of Entomology and Plant Quaratine.

J. J. de Gryse, Division of Entomology, Ottawa.

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